Immunocytochemical expression of growth factors by odontogenic jaw cysts

Tiejun Li, Roger M Browne, John B Matthews

Abstract

Aim—To determine the immunocytochemical pattern of expression of transforming growth factor (TGF) α, epidermal growth factor (EGF), and TGFβ in the three most common types of odontogenic jaw cyst

Methods—Growth factor expression was detected in paraffin wax sections of odontogenic cysts (27 odontogenic keratocysts, 10 dentigerous cysts, and 10 radicular cysts) using a streptavidin-biotin peroxidase technique with monoclonal antibodies directed against TGFα (clone 213-4.4) and TGFβ (clone TB21) and a polyclonal antibody directed against EGF (Z-12).

Results—The epithelial linings of all cysts showed reactivity for TGFα which was mainly localised to basal and suprabasal layers. Odontogenic keratocyst linings expressed higher levels of TGFα than those of dentigerous and radicular cysts, with 89% (24/27) of odontogenic keratocysts exhibiting a strong positive reaction compared with 50% (five of 10) of dentigerous and radicular cysts, respectively. EGF reactivity was similar in all cyst groups, weaker than that for TGFα and predominantly suprabasal. TGFα and EGF were also detected in endothelial cells, fibroblasts and inflammatory cells within the cyst walls. The most intense TGFβ staining in odontogenic cysts was extracellular within the fibrous tissue capsules, irrespective of cyst type.

Conclusions—These results, together with previous studies of EGF receptor, indicate differential expression of TGFα, EGF and their common receptor between the different types of odontogenic cyst, suggesting that these growth factors (via autocrine or paracrine, or both, pathways) may be involved in their pathogenesis.


Keywords: EGF; TGFα; odontogenic cysts; TGFβ.

Unit of Oral Pathology, School of Dentistry, University of Birmingham, St Chad's Queensway, Birmingham B4 6NN

Correspondence to: Dr J B Matthews.

Accepted for publication 28 October 1996

Transforming growth factor alpha (TGFα) and epidermal growth factor (EGF) are structurally related mitogens that regulate cell growth and differentiation.1 2 Both polypeptides are ligands bound by the external domain of the 170 kD epidermal growth factor receptor (EGF-R) localised to the cell surface membranes of many cell types.3 4 Ligand–receptor binding activates a tyrosine specific protein kinase which is part of the intracellular domain of the EGF-R and leads to intracellular changes preparing the cell for DNA synthesis and cell division.2 3 5 6

EGF was first isolated from mouse submandibular glands and found to accelerate incisor tooth eruption and eyelid opening in the developing animal.7 It is a potent mitogen which stimulates the growth and differentiation of a variety of mammalian cells, including epithelial cells.7 TGFα was originally isolated from retrovirally transformed 3T3 fibroblasts as a factor that stimulated anchorage independent growth.9 Like EGF, high level expression of TGFα is mitogenic in cell lines possessing EGF receptors.8 Both ligands have been identified in various human fetal and adult tissues10-11 and high levels of TGFα, and sometimes EGF, have been detected in a wide variety of neoplastic tissues including odontogenic tumours.12 13

Recent studies have suggested that members of the EGF family (that is, EGF, TGFα, and EGF-R) are involved in regulation and control of tooth development.16-18 Administration of EGF or TGFα to newborn mice accelerates incisor eruption.19 20 Exogenous EGF has been shown to inhibit early mouse tooth morphogenesis and cytodifferentiation in vitro, and to decrease tooth size in vivo.18 20-22 Furthermore, EGF antisense oligodeoxynucleotides can block murine odontogenesis in vitro.23 Although studies on EGF-R expression by the tooth germ are contradictory,19 18 22 one recent study has demonstrated that EGF-R mRNA and immunoreactive cells are mostly located in the epithelial elements of human tooth germ and its expression is subjected to temporospatial variation at different stages of tooth development.24 As different types of odontogenic cyst arise from the epithelial remnants formed at different stages of tooth development, they may reflect the cellular and molecular events occurring during normal odontogenesis, particularly in developmental cyst types. In recent studies, we have demonstrated differential expression of EGF receptors and parathyroid hormone related protein (PTHrP) by, and qualitative and quantitative differences in proliferative state between, the different types of odontogenic cyst.25-28 The aim of this study was to determine the immunocytochemical expression of TGFα and EGF in different types of odontogenic cyst. As TGFβ, a multifunctional growth factor, has also been implicated in tooth development, possibly as a signalling regulator of epithelial differentiation,29 30 its distribution in different cyst types was also studied.
**IMMUNOCYTOCHEMISTRY**

Expression of TGFα, EGF and TGFβ was detected using a streptavidin-biotin immunoperoxidase technique (StrAviGen; BioGenex, San Ramon, USA) on freshly cut, 5 μm paraffin wax sections that had been flattened onto polylysine coated glass slides at room temperature overnight. Clone 213-4.4 (OncoGene Science, Uniondale, USA), specific for amino residues 34–50 at the C-terminus of TGFα, was used at a concentration of 0.5 μg/ml. EGF was detected using a rabbit polyclonal antibody (Z12; Santa Cruz Biotechnology) raised against recombinant human EGF and used at a concentration of 2.5 μg/ml. Affinity purified monoclonal antibody directed against TGFβ (clone TB21; mouse IgG,k, Serotec, Oxford, UK), raised against purified TGFβ1 from human platelets, was used at a concentration of 0.33 μg/ml. This antibody has been shown to neutralise the action of TGFβ1 on the growth of various target cells in vitro (mink lung epithelial cells; NRK 49F cells) and to react with recombinant human TGFβ1 and purified human platelet TGFβ1 by ELISA. On western blotting, single bands corresponding to the 12.5 kD monomer and 25 kD dimer were observed from crude acid/ethanol extracts of human platelets after electrophoresis in reducing and non-reducing conditions, respectively. At the present time, possible cross-reactivity with other TGFβ isotypes and latent forms of TGFβ has not been tested.

In brief, deparaffinised sections were immersed in 0.3% hydrogen peroxide in buffer for 10 minutes to abolish endogenous peroxidase activity, washed and then overlayed with primary antibodies at room temperature for two hours. After washing, the sections were further treated with biotinylated multi-link (BioGenex, 1 in 75 dilution in buffer containing 10% normal human serum; one hour at room temperature), washed and then overlaid with peroxidase-labelled streptavidin (BioGenex, 1 in 75 dilution; one hour at room temperature). Bound peroxidase was visualised by immersing washed sections in 3,3'-diaminobenzidine (five minutes) and reaction products subsequently enhanced by treatment with 0.5% copper sulphate (w/v in saline; five minutes). Stained sections were lightly counterstained in Meyer's haematoxylin and mounted in Xam. Phosphate buffered saline (PBS; 0.01 M; pH 7.6) was used for all reagent dilutions and washes.

**Methods**

**TISSUES**

Forty seven odontogenic cysts (27 odontogenic keratocysts, 10 dentigerous cysts, and 10 radicular cysts) were retrieved from the files of the Unit of Oral Pathology. Of the 27 odontogenic keratocysts, 10 were primary, occurring as a solitary lesion in otherwise healthy patients; eight cysts were recurrent lesions within a period of three to 10 years after primary surgery; nine cysts were from six patients known to have naevoid basal cell carcinoma syndrome (NBCCS). All tissue specimens were fixed in 10% neutral buffered formalin (18–48 hours), processed routinely and embedded in paraffin wax. Only undecalcified specimens or specimens that had been decalcified in 10% formic acid for less than six hours were chosen to minimise possible inconsistencies in immunoreactivity as a result of such processing variables. Routinely fixed and processed specimens of human skin, salivary glands and oral squamous cell carcinomas were used as positive controls.

**Table 1** Immunoreactivity of odontogenic cyst epithelium with antibodies directed against TGFα and EGF

<table>
<thead>
<tr>
<th>Epithelial lining</th>
<th>Number of specimens</th>
<th>TGFα</th>
<th>EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Odontogenic keratocyst</td>
<td>27</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Solitary</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Recurrent</td>
<td>8</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Syndrome</td>
<td>9</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Dentigerous cyst</td>
<td>10</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Radicular cyst</td>
<td>10</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

*Number of cases showing degree of reaction: ++, strong; +, weak; −, no staining.
†Two specimens each of dentigerous and radicular cysts were EGF negative.
EGF, TGFα and TGFβ in odontogenic cysts

Optimum dilutions, times and temperatures were determined in preliminary experiments on a variety of routinely fixed and processed tissue blocks. Positive staining was abolished when normal mouse IgG, a monoclonal antibody of irrelevant specificity (MRC OX-52, IgG2a anti-rat T cell; Serotec) or PBS were used as the first layer and when one or both second layers were omitted. The specificity of the reaction was determined by preincubation of antibodies with 10-fold excess by weight of their corresponding peptides at 4°C overnight (TGFα, Sigma, Poole, Dorset, UK; EGF, Santa Cruz Biotechnology; TGFβ, Serotec).

EVALUATION OF TISSUE SECTIONS

Overall, TGFα, EGF and TGFβ staining intensity of different cyst linings was scored subjectively using a semiquantitative scale: strong (++), weak (+) and negative (−). In addition, the distribution of TGFα and EGF staining within the cyst linings was scored to indicate which epithelial layer(s) exhibited the strongest reaction: basal and suprabasal; and suprabasal. Because substantial staining of TGFβ was also seen in the fibrous tissue capsules of odontogenic cysts, its staining intensity was assessed subjectively for both cyst

Figure 2  Staining of odontogenic keratocysts (A and D), dentigerous (B) and radicular cysts (C), and epithelial rests within the connective tissue wall (E) for TGFα. Note the staining pattern of strong basal and granular layer reactivity (D) in some odontogenic keratocyst linings, and strong TGFα reactivity in connective tissue wall associated with the presence of inflammation (F).

Figure 3  Staining of an odontogenic keratocyst (A) and a dentigerous cyst (B) for EGF.
linings and fibrous tissue capsules. To assess objectively the immunoreactivity of TGFα and EGF in odontogenic cyst linings, the absorbance of the stained cyst linings was determined, at a magnification of ×200, using TV image analysis (Seescan Prism; Seescan Imaging, Cambridge, UK). This image analysis system gives true, reproducible absorbance readings that are independent of the level of background illumination and a linear response over a wide range of absorbance values (0 to +3).

Preliminary titration experiments with EGF and TGFα antibodies showed that the chromogen density obtained at working concentrations was within this linear response range. Measurements were made in five areas of cyst lining in each case. Data were analysed statistically using the Mann-Whitney U-test.

## Results

### STAINING PATTERN IN CONTROL TISSUES

TGFα and EGF staining was localised to the cytoplasm and mainly detected in epidermis, ducts of salivary glands and epithelial tumour components. In epidermis and oral squamous cell carcinoma, strong reactivity for TGFα was usually detected in areas showing squamous differentiation and in central areas of tumour nests. Staining in the basal cell layers was weak or absent (fig 1A). TGFα positive staining was also found in the cytoplasm of endothelial cells, striated and smooth muscle, macrophages, plasma cells, and fibroblasts. Overall, staining intensity of TGFα was stronger than that of EGF. By contrast, the most intense staining for TGFβ was found in the connective tissues of the positive control specimens and within the epithelium, it was extracellular and usually weaker. However, the staining reactions of all three antibodies were completely abolished by preincubation with excess corresponding recombinant growth factor (fig 1).

### TGFα AND EGF STAINING IN ODONTogenic CYSTS

Table 1 summarises overall TGFα and EGF staining intensity and pattern of distribution within the epithelial linings of odontogenic cysts. The epithelial linings of all cysts showed reactivity for TGFα (fig 2) with those of odontogenic keratocysts exhibiting a stronger reactivity than dentigerous and radicular cysts. Generally, the distribution of TGFα staining did not differ between cyst types: most exhibited predominantly basal and suprabasal reactivity. However, in areas of 10 odontogenic keratocyst linings, strong staining was seen in the basal and upper cellular layers, with weaker reactivity between the two (fig 2D). There was no detectable difference between the three subgroups of odontogenic keratocyst (that is, solitary, recurrent, and NBCCS-associated cysts) both in terms of epithelial staining intensity and distribution. TGFα and EGF were also detected in endothelial cells, fibroblasts, and inflammatory cells within the cyst capsules. The staining intensity of these cells for TGFα seemed to be higher in areas associated with inflammatory foci. In odontogenic keratocysts, there was no apparent alteration in TGFβ reactivity exhibited by disordered cyst lining associated with areas of inflammation (fig 2F). Epithelial rests also stained strongly for TGFα (fig 1E). In all specimens, EGF reactivity was weaker than that for TGFα, and two specimens each of dentigerous cyst and radicular cyst were negative. The distribution

---

Figure 4 Histogram showing TGFα and EGF absorbance readings of the epithelial linings of odontogenic cysts measured by TV image analysis (mean absorbance (SD)). Rec = recurrent, Syn = syndrome, OKC = odontogenic keratocyst.

Figure 5 Odontogenic keratocyst (A), dentigerous cyst (B) and radicular cyst (C) stained for TGFβ.
of EGF was also different from that of TGFβ, being predominantly suprabasal (fig 3).

Absorbance measurements for TGFα and EGF expression in different cyst linings, determined by TV image analysis, are summarised in fig 4, and confirm the subjective assessments. The mean values for TGFα were significantly higher than those for EGF (p < 0.004) and TGFα absorbance readings for all three subgroups of odontogenic keratocyst were significantly higher than for dentigerous cysts (p < 0.05). Radicular cysts showed great variation in TGFα absorbance and were only significantly different from the NBCCS odontogenic keratocyst group (p < 0.02). This may be partly because of the greater structural variability of radicular cyst linings (for example, intercellular spaces and areas of necrosis) which will affect readings. There were no significant differences in EGF absorbance readings between different cyst types. Within the odontogenic keratocyst group, the only significant difference in TGFα absorbance readings was between NBCCS-related lesions and solitary cysts (p < 0.05).

**Expression of TGFβ in Odontogenic Cysts**

In contrast to TGFα and EGF expression in odontogenic cysts, the most intense staining of TGFβ was found in the fibrous tissue capsules, irrespective of cyst type, with the majority of cyst linings exhibiting patchy weak reactivity (fig 5). The epithelial staining appeared to be intercellular and predominantly suprabasal. Consistent strong TGFβ staining was detected in both cellular (fibroblasts, endothelial and inflammatory cells) and intercellular (matrix, intravascular spaces) components of the connective tissue walls in all cyst types. In some sections, a stronger intercellular reactivity was found in the peripheral part of the tissue with reactivity becoming relatively weaker towards the centre.

**Discussion**

Our results on control tissues are consistent with published studies using the same antibodies on paraffin wax sections. Furthermore, the peptide inhibition studies confirm the specificity of the staining reactions obtained with all three growth factor antibodies on both control and odontogenic cyst specimens.

The results show that epithelial linings of all types of odontogenic cyst express TGFα, although the intensity of staining varied between cyst types, with odontogenic keratocyst linings exhibiting more intense reactivity than those of dentigerous and radicular cysts. This is consistent with the higher levels of EGF-R expression, and proliferating cell nuclear antigen (PCNA) and Ki-67 labelling indexes in odontogenic keratocyst epithelium which suggest that these cysts have an intrinsic growth potential not present in the other types of odontogenic cysts. It has been suggested that coexpression of TGFα and EGF-R in neoplastic cells gives them a growth advantage over normal cells as antibodies directed against TGFα or EGF-R antisense oligonucleotides have been shown to inhibit the proliferation of several carcinoma cell lines. The strong TGFα reactivity coincident with the high levels of EGF-R expression in odontogenic keratocyst linings suggest that TGFα may act as an autocrine growth factor to stimulate cell proliferation or differentiation, or both, in this cyst type. That such a relation may exist is also suggested by the significantly higher levels of TGFα detected in NBCCS-odontogenic keratocysts compared with solitary odontogenic keratocysts as it is known that syndrome-related cysts exhibit higher mitotic and Ki-67 labelling indexes.

Our previous studies have shown that dentigerous cyst linings exhibit a high level of EGF-R expression and that their staining intensity is similar to that of the odontogenic keratocyst. However, TGFα staining in dentigerous cyst linings was patchy and weak and its absorbance was significantly lower than that of odontogenic keratocysts (p < 0.05). This differential expression of TGFα and its receptor EGF-R by dentigerous cyst linings may indicate that paracrine mechanisms of growth factor control are a more important feature in this type of developmental cyst. It is interesting to speculate that this may be directly or indirectly associated with the apparent indolence of the epithelial linings of this cyst type, which have significantly lower PCNA and Ki-67 labelling indexes.

In all cyst types, foci of inflammation in the fibrous capsule were associated with areas of strong stromal TGFα reactivity (fibroblasts, inflammatory and endothelial cells) with no apparent difference in the reactivity of the overlying cyst lining. Interestingly, previous studies have shown an inverse relation between the presence of inflammation and the staining intensity of adjacent overlying epithelium for EGF-R. Such differences may reflect epithelial–mesenchymal interactions and growth factor/receptor modulation. It is believed that such processes play an important role in modulating cell growth and cell interaction during normal odontogenesis as differential expression of EGF-R and TGFα has been shown to relate to the stages of tooth development in both dental epithelium and dental mesenchyme.

EGF immunoreactivity in the epithelial linings of odontogenic cysts was patchy and weaker than that for TGFα, with no detectable differences in staining intensity between cyst types. This difference in reactivity was also a feature of the staining results on control tissues. Similar weak or negative EGF reactivity in conjunction with consistent positive TGFα staining has been reported in various normal tissues and tumours, including odontogenic tumours and oral squamous cell carcinoma. Thus, odontogenic cyst linings are similar to other tissues in that TGFα seems to be the key factor in the potential autocrine loop for stimulation of EGF-R. However, it is important to realise that differences in staining intensity obtained with two different antibodies, for the same or different antigens, do not
necessarily equate with true quantitative differences in amount of antigen within the section. Moreover, immunochemical studies of TGFβ1 have relied on two rabbit polyclonal antibodies (anti-CC and anti-LC) directed against unconjugated peptides corresponding to the N-terminal 30 amino acids of TGFβ1. The antibodies recognise different epitopes: anti-CC shows predominantly extracellular (mesenchymal) and anti-LC predominantly intracellular (epithelial) reactivity. The close correlation of intracellular staining using anti-CC with in situ localisation of TGFβ1 mRNA suggests that anti-CC recognises TGFβ1 at sites of synthesis (latent or precursor form of TGFβ1) whereas anti-CC may detect the active form of TGFβ1 which is bound to extracellular matrix proteins. In this study, the pattern of monoclonal antibody TB21 staining resembles that of anti-CC, mainly showing extracellular and mesenchymal reactivity on both control and odontogenic cyst specimens. Although the isotype specificity of TB21 is not known, its ability to neutralise the action of TGFβ1 on the growth of various target cells in vitro suggests that it reacts with the active form of TGFβ1. Therefore, our results may indicate the ubiquitous presence of the active form of TGFβ1 in the fibrous tissue capsules of all odontogenic cyst types. However, it is not possible to know whether this is because of locally produced or systemically derived peptide or a combination of both. Further studies are required using isotype specific reagents (antibodies and oligonucleotides/riboprobes) to characterise the TGFβ detected and assess the role of active local synthesis.

In conclusion, our results show the presence and differential expression of TGFβ and EGF in the epithelial linings of odontogenic cysts. Taken together with our previous data on EGF-R expression, these results suggest that members of the EGF family (via autocrine or paracrine, or both, pathways) are important and play a differential role in the initiation and growth of odontogenic cysts.

We would like to thank the West Midlands Regional Health Authority for financial support and Mrs Christine Wilson for excellent technical assistance. T.J.L. is partly supported by an Overseas Research Study award.

36. Sizeland AM, Burgess AR. The proliferation of morpho- logic responses of a colon carcinoma cell line (LIM 1215)


